EFFECT OF VITAMIN B_6 AND CALCIUM PANTOTHENATE ON THE METABOLISM OF UNSATURATED FATTY ACIDS

by

WU-CHIEH CHENG

B. S., St. John's University
Shanghai, China, 1944

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1949

Document
LD
2668
T4
1949
C49
c.2
7-18-57 D.R.

TABLE OF CONTENTS

INTRODUCTION 1
EXPERIMENTAL 4
EXPERIMENTAL PROCEDURE 5
    Preparation of Supplements 5
    Saponification Method 10
    Iodine Number Determination 11
    Spectrophotometric Analysis 13
RESULTS 16
DISCUSSION 30
SUMMARY 34
ACKNOWLEDGMENT 36
REFERENCES 37

Neenah
CHIEFTAIN BOND
RAG CONTENT
MADE IN U.S.A.

INTRODUCTION

It has been established that animals need "essential" fatty acids for normal growth. Burr and Burr (8, 9) showed that if young animals were subjected to a complete fat starvation over a period of several months, they developed dermatosis, and soon died. These authors reported that the presence of highly unsaturated fatty acids, such as linoleic and linolenic acids, in the diet was essential for the normal growth of rats. Later in 1938, Turpeinen (31), and Nunn and Smedley-MacLean (17) stated that arachidonic acid was also an essential fatty acid.

By the use of deuterium as an indicator, Schoenheimer and Rittenberg (28) found that deuterostearic acid was dehydrogenated by rats to oleic acid, and Stetton and Schoenheimer (30) showed that the animals can desaturate palmitic acid to palmitoleic acid. The desaturation cannot go further than oleic acid, since the linoleic acid isolated from these rats contained no significant quantity of deuterium.

The relative power of the essential fatty acids in the curing of the fat-deficient disease has been studied by various workers. Burr (7) stated generally that both linoleic and linolenic acids were very effective curative agents. Meanwhile linoleic acid was reported to be 6 times more potent than linolenic acid by Hume et al. (13), and arachidonic acid to be at least approximately 3 times as effective as linoleic acid by Turpeinen (31). On the other hand, Quackenbush, Kummerow and

Steenbock (19) claimed that linolenic acid was nonfunctional in reproduction.

In fat metabolism, some of the vitamin B complexes have been shown to be functional. Among them pyridoxine (or vitamin B_6) and pantothenic acid seem to have special importance. Birch (3) found that, in the absence of an adequate supply of pyridoxine, the animals were unable to make proper use of the unsaturated fatty acids, or alternately, in the absence of adequate supply of unsaturated fatty acids, the animals were unable to utilize its pyridoxine. The same conclusion was drawn by Salmon (26) who stated that, although the pyridoxine and the essential fatty acids can to a certain extent mutually alleviate the deficiency of each other, the presence of both in the diet was necessary for normal nutrition of rats.

In 1940, Oleson and Black (18) found that the chick antidermatitis factor (pantothenic acid) was required by the rat. Recently, Lipmann (14) suggested that a pantothenic acid-containing enzyme participates generally in protein and fat synthesis and other processes requiring the activation of a carboxyl group as a preliminary to condensation.

Some work has been done to differentiate the effect of the essential fatty acids and the B-vitamins in the curing of dermatitis of rats. Schneider et al. (27) suggested that pyridoxine and the essential fatty acids were interchangeable. However, Quackenbush et al. (22) have shown the above suggestion to be invalid, and indicated that both pyridoxine and pantothenic acid were necessary to render the proper utilization of the essential fatty acids. However, they were unable to determine how these factors were involved in fat-metabolism.

The rate at which rancidity developed was found to be dependent on the presence of highly unsaturated fatty acids in the depot fats of the carcasses of turkeys and chickens, which were held in cold storage for an extended period of time (12). This is in agreement with the results obtained by Barnes, Lundberg, Hanson and Burr (2), who studied the natural stability of body fat from rats by changing the dietary supplements. Furthermore, Anderson and Mendel (1) have shown that the properties of the depot fats of animals were dependent upon the nature of the dietary fats. It is the purpose of the present research to find out the correlation between the dietary fats and the depot fats of the animals.

In the present study, the metabolism of the esters of oleic, linoleic, linolenic, arachidonic and the saturated fatty acids from cottonseed oil was studied. These esters were also fed to rats which had received additional supplements of pyridoxine and pantothenic acid. The composition of the carcass and liver fat of the rats, which had received the different diets, were studied by means of the spectrophotometric method of analysis.

EXPERIMENTAL

Rats of the Sprague-Dawley strain between 50 to 60 g in weight and three weeks of age were transferred to individual wire cages and given water and Diet V ad libitum. Diet V (Table 1) of Quackenbush, Platz and Steenbock (20), which is free of pyridoxine, pantothenic acid and fat, was prepared as follows: 114.6 g of cystine, 366.7 g of glycine, 45.5 g of inositol, 91.0 g of choline chloride, 4550 mg of niacin, 200 mg of thiamin, 400 mg of riboflavin, 136.5 mg of paraminobenzoic acid, 36.4 mg of folic acid, and 475 ml of biotin were dissolved in N/50 acetic acid, mixed with 18 pounds of casein, dried and then mixed with 78 pounds of cerelose and 4 pounds of Wesson salts in a standard feed mixer. The animals were supplemented once each week with one drop of fish liver oil containing 250 IU of vitamin A and 100 IU of vitamin D per drop.

After four weeks, the rats were divided into 11 groups of 4 to 6 each and fed with the aid of a calibrated medicine dropper the supplements listed in Table 2. The ethyl esters of arachidonic, linolenic, and linoleic acid were prepared by the debromination (Rollet, 25). Methyl oleate was made according to the method of Brown and Shinowara (6), and the ethyl esters of saturated acids were prepared by direct esterification of the saturated fatty acids from cottonseed oil with ethyl alcohol. The resulting esters were distilled under high vacuum, and kept under an atmosphere of nitrogen at -13 degrees centigrade.

Water soluble supplements were prepared by dissolving them in water. After five weeks of supplementation, the animals were killed and saponified with Potassium hydroxide as described by Quackenbush and Steenbock (21). The composition of the mixed fatty acids was determined spectrophotometrically according to Brice et al. (5), and the iodine numbers were determined according to the method of Yasuda (32). All methods were outlined under experimental procedures.

The percentages of individual fatty acids, calculated from spectrophotometric analysis, were converted to mg of fatty acid per 100 g of tissue. Both of these values were used; the formula used for the conversion was:

$$\text{mg}/100\ \text{g} = (\%\ \text{fatty acids})(M)(1000)/(100),$$

where M equals to mean weight of extracted fatty acid.

EXPERIMENTAL PROCEDURES

Preparation of Supplements (25, 6)

Preparation of Ethyl Linoleate. Three hundred grams of potassium hydroxide were placed in a five-liter round-bottom flask, into which 1200 ml of ethyl alcohol and 100 ml of water were added. After the mixture was heated to near boiling on a steam bath, 1000 grams of fresh cottonseed oil were added. With a reflux condenser attached, the mixture was allowed to reflux for 30 minutes. The hydrolysate was cooled to about 40 degrees centigrade in a stream of tap water and 1300 ml of cold distilled water were added. With continued cooling and agitation, 550 ml of cold concentrated hydrochloric acid were added in small portions. The cold mixture was then transferred to a large separatory funnel and shaken vigorously to insure complete decomposition of the soaps. The fatty acids were washed twice with about one liter of distilled water, care being taken to avoid emulsification. One liter of redistilled petroleum ether was added and the fatty acids were washed again. After the water had been drawn off the fatty acids were placed in a five-liter round-bottom flask to which about 50 g of anhydrous sodium sulphate was added. One liter of redistilled petroleum ether was then added and the solution was allowed to stand overnight at -5 degrees centigrade. The saturated acids and the sodium sulphate were filtered off and one liter of redistilled petroleum ether was added to the filtrate which was then ready for bromination.

The five-liter flask containing the solution of fatty acids was clamped firmly into an ice-salt bath, the flask being at least three inches above the bottom of the bath to provide proper cooling. A mechanical stirrer with sufficient speed and power to produce good mixing was adjusted to about one-half inch from the bottom of the flask. Bromine was added from a separatory funnel at such a rate that the temperature of the reaction mixture at no time exceeded 10 degrees centigrade. About 580 g of bromine were required for complete saturation, as indicated by the persistence of a bromine color. The flask was corked tightly and allowed to stand overnight at -5 degrees centigrade. The crystalline crude tetrabromostearic acid was collected on a Buchner funnel, washed with redistilled petroleum ether and transferred to a dry five-liter round-bottom flask. Five liters of redistilled petroleum ether and 1.5 liters of ether, or more if necessary to effect complete solution, were added and the tetrabromostearic acid was brought into solution by refluxing on a steam bath. With the addition of 20 g of Norit the solution continued to reflux for a few minutes more, and then the hot solution was filtered through a warm Buchner funnel. Oversized filter paper was used in the funnel and was kept firmly in place by a water bath ring which fitted snugly in the funnel. The filtrate was allowed to stand overnight at -5 degrees centigrade. The product was filtered on a Buchner funnel and washed with redistilled petroleum ether. The white crystals were dried at room temperature and their melting point was determined. If the melting point was low, the product was recrystallized from petroleum ether again before proceeding.

Two hundred g of tetrabromostearic acid and 200 g of granular zinc were mixed together and placed in a dry, one liter round-bottom flask. A condenser was attached and 200 ml of absolute ethyl alcohol were added and the acid dissolved by warming carefully on a steam bath. Thirty ml of 3-normal solution of dry hydrochloric acid in absolute alcohol were cautiously added, and the flask was carefully rotated to start the reaction. Cooling in a stream of tap water was necessary to control the initial reaction, after which the mixture was shaken vigorously and allowed to stand until the esters had separated completely. The aqueous phase was then drawn off and discarded and the esters washed with an equal volume of distilled water, care being taken to avoid emulsification. After being dried with anhydrous sodium sulphate, the esters were distilled under vacuum. The distillate was sealed in 10 ml bulbs under high vacuum.

Preparation of Ethyl Linolenate. Linseed oil was used for the raw material. The same directions described above were followed except that the fatty acids obtained were brominated in ether instead of petroleum ether, and that the recrystallization medium for the hexabromostearic acid was benzene instead of petroleum ether.

Preparation of Ethyl Arachidonate. Beef adrenal phosphatide was used as raw material. The same directions for the preparation of ethyl linoleate were employed with a few modifications. Acetone was used to dissolve the fatty acids, and the solution was cooled to -70 degrees centigrade with dry ice and acetone. The precipitated fatty acids were separated by filtration through a Buchner funnel. The filtrate was esterified with ethyl alcohol, and the ester was extracted with ethyl ether. The ether solution was brominated at zero degree centigrade. The precipitate obtained was filtered, washed with cold ether, and debrominated with zinc as described above.

Preparation of Methyl Oleate. One kilogram of olive oil was saponified and the soaps converted into fatty acids as mentioned above. Two hundred and twenty five g of the fatty acids were dissolved in 3450 ml of acetone in a four-liter flask and allowed to stand in the cold room overnight. The precipitate was removed by suction-filtration on a large Buchner funnel. The filtrate was cooled to -60 degrees centigrade and filtered. After filtering, the precipitate was allowed to melt and was made up to 2000 ml with acetone, and again brought to -60 degrees centigrade and filtered. After two more crystallizations at this temperature, the final precipitate was melted, made up to 1250 cc with acetone, and cooled slowly with stirring to the point of first appearance of crystals, which was about -35 degrees centigrade. These crystals consisted of oleic acid and any saturated acids which might have escaped the first crystallization. They were filtered off in the cold room, and the acetone was removed under reduced pressure. The residual product was esterified with methyl alcohol in the presence of about 2 percent of concentrated sulphuric acid, and the unesterified acids were removed by washing with dilute potassium carbonate solution. The methyl oleate obtained was distilled over under reduced pressure.

Preparation of Ethyl Esters of Saturated Fatty Acids. In the preparation of linoleic acid from cottonseed oil, saturated acids were filtered off from petroleum ether solution at -5 degrees centigrade. This mixture of saturated fatty acids was esterified with ethyl alcohol with about 2 percent of concentrated sulphuric acid as a catalyst. The unesterified acids were removed by washing with dilute potassium carbonate solution. The resulting esters showed an iodine number of 7.84 on analysis. Spectrophotometric analysis showed that they were composed of 8.16 percent of ethyl oleate and 91.84 percent of saturated acids esters.

Saponification Method (21)

Animals were weighed and those of the same group were combined. They were killed with ethyl ether. Carcass and liver tissues were separated and analyzed.

The carcasses or livers were weighed and allowed to digest with 100 ml of 30 percent KOH solution per 100 g of tissue on a steam bath for two hours. About 100 ml of 95 percent ethyl alcohol were added to the hydrolysate which had been freed of the bones. The mixture was allowed to reflux for about two hours until all the fatty materials were completely saponified.

Redistilled Skellysolve F was employed to take off the non-saponifiable materials from the mixture by extration. The aqueous layer was acidified with concentrated hydrochloric acid and the liberated fatty acids were extracted with redistilled Skellysolve F. Excess hydrochloric acid in the extract was washed out with water, and the extract was dried over anhydrous sodium sulphate. After the drying agent had been separated by filtration, the solvent was removed by distillation under reduced pressure. The fatty acids obtained were weighed and the percentages of fatty acids in carcass or liver of the animals was calculated from the following formula:

$$\%\ \text{Fatty Acids} = \frac{(\text{Wt. of fatty acids obtained})(100\%)}{(\text{Wt. of carcass or liver})}$$

Iodine Number Determination (32)

Approximately 0.1 g of fatty acids was weighed accurately into a glass stoppered iodine flask. With a volumetric pipette, exactly 15 ml of Wijs solution were added. The flask was stoppered and placed in the dark for exactly one hour. Then 10 ml of potassium iodide solution were added, the mixture shaken vigorously and the stopper and sides of the flask washed with 10 ml of distilled water. The mixture was titrated with 0.1 N thiosulphate solution, to faint yellow and then to colorless after the addition of a few drops of starch indicator. Two blank determinations were run in the same manner along with unknowns.

The solutions used above were prepared as follows:

Wijs Solution. Thirteen grams of powdered resublimed iodine were dissolved in 1000 ml of pure glacial acetic acid which was warmed to facilitate the solution of the iodine. After being cooled to room temperature, 10 ml of the iodine solution was taken out, mixed with 80 ml of water, and titrated with the standard thiosulphate solution with starch solution as an indicator. About 30 ml of the iodine solution was put aside, and dry chlorine gas was passed into the remainder until the color of the solution did not appear to change. Ten ml of this solution was mixed with 10 ml of the potassium iodide solution and 80 ml of water, and titrated as before with the standard thiosulphate solution. Sufficient chlorine should be added to double the titration of the original iodine solution, but little chlorine in excess of this requirement could be present. The titration of 10 cc portions of the solution after each further addition of chlorine was continued until the halogen content of the solution was doubled. Then the reserved 30 ml portion of the iodine solution was added and mixed thoroughly. The solution was kept in glass-stoppered bottle and stored in the dark.

Potassium Iodide Solution. Fifteen grams of potassium iodide were dissolved in 85 ml of distilled water.

Thiosulphate Solution. Twenty-four and 8 tenths g of sodium thiosulphate were dissolved in one liter of water. The exact normality was determined by standardization against standard potassium dichromate solution as follows: 10 ml of 0.1 N potassium dichromate solution was measured out accurately, and 5 ml of concentrated hydrochloric acid was added. After being mixed with 10 ml of 15 percent potassium iodide solution, it was titrated with 0.1 N thiosulphate solution. The starch solution was added when the solution turned greenish. The flask was well shaken during titration, as the end point was reached suddenly. The titration was continued until the solution showed a clear, bright green, or until one drop of 0.1 N thiosulphate would not alter the color.

The formulas used in the calculation of iodine numbers were:

Normality factor equals to (126.9 x 0.1) /cc of thiosulfate.

Iodine number equals to (Normality factor x D)/ sample wt., where D is the difference between the volumes in cc of the thiosulphate solutions used in the titrations of the sample and the blank.

Spectrophotometric Analysis (5)

About 0.1 g of fatty acids was weighed accurately into a small weighing vessel which was then placed each in a marked ignition tube. Two blanks were run with the samples at the same time. To each tube, 4 ml of ethylene glycol which contained 7.5 percent KOH was added, and nitrogen was continuously blown in for the displacement of air over the sample. The tube was placed in wire basket, and heated in oil-bath at 180 degrees centigrade (within two degrees centigrade fluctuation) for half an hour.

After the heating, the tube was removed from the basket, and the oil wiped off. The content of each tube was transferred quantitatively to a 100 ml volumetric flask, and diluted to the mark with absolute ethyl alcohol, some of which was used in washing the ignition tube and the funnel during the transfer. The volumetric flask was shaken well to insure a good mixing, and allowed to stand for 5 to 6 hours or overnight in a cold room to allow the silica from glass to precipitate. Then the samples were brought to room temperature, and the silica precipitate was filtered off. The first 15 to 20 cc of filtrate, which saturated the filter paper and washed out the contaminating substances present on the filter paper, was discarded. After the rest of the sample had been filtered, 10 cc of the filtrate was carefully pipetted into a 200 ml volumetric flask. The pipette was washed with absolute ethyl alcohol between each sample and rinsed with each sample before use. Absolute ethyl alcohol was used to dilute the sample to volume. The sample, after having been shaken well, was read on Beckman Spectrophotometer, and readings of logarithm Io/I or extinction were recorded at the wave-lengths of 2330, 2340, 2620, 2680, 2740, 3100, 3160 and 3220 A. If the sample was too concentrated to read at the above wave-lengths, suitable dilutions were made, and blanks were diluted in the same way. As correction-factors in the calculation, the unisomerized sample in the concentration of 0.1 g in 100 ml of absolute ethyl alcohol was read on Beckman spectrophotometer at the stated wave-lengths.

The calculation was carried out with the following formulas:

$$K_2 = (K_{2330} \times 10) / (4 \times \text{sample wt.}) - 0.07$$

$$K_3 = (2.8 \times 10)(K_{2680} - \tfrac{1}{2}(K_{2620} + K_{2740})) / (4 \times \text{sample wt.})$$

$$K_4 = (2.8 \times 10)(K_{3150} - \tfrac{1}{2}(K_{3100} + K_{3220})) / (4 \times \text{sample wt.})$$

$$K'_2 = (K'_{2330} \times 10) / (4 \times \text{sample wt.}) + 0.04$$

$$K'_3 = (4.1 \times 10)(K'_{2680} - \tfrac{1}{2}(K_{2620} + K'_{2740})) / (4 \times \text{sample wt.})$$

$$K'_4 = (2.8 \times 10)(K'_{3150} - \tfrac{1}{2}(K'_{3100} + K'_{3220})) / (4 \times \text{sample wt.})$$

$$K''_2 = K'_2 - K_2$$

$$K''_3 = K'_3 - K_3$$

$$K''_4 = K'_4 - K_4$$

$$\%\ \text{Linoleic acid} = 1.125 \times K''_2 - 1.107 \times K''_3 + 0.04 \times K''_4$$

$$\%\ \text{Linolenic acid} = 1.87 \times K''_3 - 4.45 \times K''_4$$

$$\%\ \text{Arachidonic acid} = 4.43 \times K''_4$$

$$\%\ \text{Oleic acid} = (\text{Iodine number} \times 100 - A - B - C)/90$$

where $A = 181.5$ (% Linoleic acid),

$B = 273.5$ (% Linolenic acid),

$C = 333.5$ (% Arachidonic acid).

All K values were calculated from the readings on unisomerised samples, while K' values were obtained from those on isomerised samples.

The concentration of the sample solution was assumed in the above formulas to be as follows: sample was dissolved in ethyl alcohol, the solution was diluted to 100 ml, and 10 ml of this solution was diluted to 250 ml with ethyl alcohol. If samples must be diluted further in order to get readings on the Beckman spectrophotometer, the formulas should be modified accordingly. For example, if a 5 ml aliquot were taken from the 250 ml volumetric flask and diluted to 25 ml, then the sample weight must be multiplied by 2 instead of 4, and the sample reading multiplied by 100 in place of 10.

$$K'_B = (K'_{2320} \times 100) / (8 \times \text{sample wt.}) + 0.04.$$

In the calculation, any K or K' value which came to a minus value was considered as zero.

RESULTS

The presence of pyridoxine and calcium pantothenate, rather than the nature of the different fatty acids, seemed to have the most influence on the normal growth of the animals. The animals, which received pyridoxine and calcium pantothenate in the diet were found to grow normally with a rapid increase in body weight (about 70 g in 6 weeks), while those without pyridoxine and calcium pantothenate did not show any appreciable increase in body weight.

From the data obtained (Table 3), it was shown that, in general, the percentage of the mixed fatty acids was between 9 and 10 percent when pyridoxine and calcium pantothenate were fed, and between 2 and 3 when the diet was not supplemented with pyridoxine and calcium pantothenate. One group of rats was fed with a diet free of any fatty material and was not supplemented with pyridoxine and calcium pantothenate. The analyses indicated the deposition of 1.94 percent of the mixed fatty acids in the carcass tissues.

The iodine values of the mixed fatty acids from the carcass tissues were influenced by the presence or absence of pyridoxine and calcium pantothenate in the diet. In the absence of the latter, the iodine values varied from 89 to 96, and these values decreased to about 80 when pyridoxine and calcium pantothenate were fed to the animals as supplements.

The composition of the mixed fatty acids was determined by means of a Beckman spectrophotometer. The amounts of arachidonic and linoleic acids in 100 g of carcass tissues were found to remain constant and were independent of the dietary supplements of pyridoxine and calcium pantothenate (Table 4). On the other hand, the amounts of oleic acid were increased from 4736-6380 mg, and the amounts of saturated fatty acids from 3384-3708 mg per 100 g of tissue. Linolenic acid was absent in both cases.

Although the nature of the deposited fatty acids in the carcass tissues was influenced by the presence of pyridoxine and calcium pantothenate in the diet, the composition of the fatty acids in the liver remained unaffected. The liver tissues contained from 0.77 to 1.06 percent of mixed fatty acids, which consisted of 51 to 59 percent of saturated and oleic acids, 11 to 18 percent of linoleic acid, 26 to 35 percent of arachidonic acid, and no linolenic acid. This composition remained constant as long as the animals received one of the essential fatty acids in the diet, and was not affected by pyridoxine and calcium pantothenate. However, when the animals received only the basic diet, or were supplemented with methyl oleate or the ethyl esters of the saturated acids, the percentage of saturated fatty acids and oleic acid was found to be between 75 and 88 percent, and percentages of linoleic and arachidonic acids were decreased from 14.72 to 9.17 percent, and from 31.11 to 15.24 percent, respectively.

Three groups of the animals fed ethyl linolenate were used to test whether pyridoxine or calcium pantothenate was more effective (Table 6). Pyridoxine alone increased the mean body weight of the animals by 64.5 g, while a combination of pyridoxine and calcium pantothenate gave an increase of 75.7 g. In the carcass tissues of these animals, the percentage of mixed fatty acids was increased from 2.66 to 6.65 percent with pyridoxine alone, or to 11.01 percent when both pyridoxine and calcium pantothenate were present in the diet. The iodine values of the mixed fatty acids were also affected. The animals not receiving pyridoxine or calcium pantothenate gave an iodine value of 96.0 for the mixed fatty acids, those getting pyridoxine alone yielded fatty acids with an iodine value of 76.6, and those receiving both pyridoxine and calcium pantothenate had the lowest value or 73.0.

The compositions of the mixed fatty acids of the experimental animals were also compared with each other on a weight basis (Table 7). The data indicated that dietary supplementation with pyridoxine raised the amount of saturated acids from 352 mg to 1022 mg, and that of oleic acid from 2070 mg to 4830 mg. Supplementation with pyridoxine plus calcium pantothenate doubled the weight of saturated and oleic acids in the mixed fatty acids of the carcass tissues. However, the amount of linoleic and arachidonic acids were not changed to any appreciable value.

Liver tissues contained more arachidonic and linoleic acids and less oleic and saturated acids than the carcass tissues. Although supplementation with pyridoxine and calcium pantothenate influenced the composition of the mixed fatty acids in the carcass tissues, the above general statement is still true.

Table 1. Diet V.

Ingredients	Amount per 100 lbs. ration
Cerelose	78 lbs.
Casein	18 lbs.
Wesson salts	4 lbs.
Cystine	114.66 g
Glycine	366.75 g
Inositol	45.5 g
Choline chloride	91.0 g
Niacin	4550.0 mg
Thiamin	200.0 mg
Riboflavin	400.0 mg
Para-amino-benzoic acid	136.5 mg
Folic acid	36.4 mg
Biotin	19.0 cc

Composition of Wesson salts

Sodium chloride	105.00 g/Kg
Potassium chloride	120.00 "
Calcium diphosphate	149.00 "
Potassium hydrogen phosphate	310.00 "
Calcium carbonate	210.00 "
Magnesium sulphate	90.00 "
Potassium aluminum sulphate.$24H_2O$	0.09 "
Ferric pyrophosphate	59.50 "
Manganese sulphate	0.20 "
Cupric sulphate.H_2O	0.39 "
Potassium iodide	0.05 "

Table 2. Supplements to diet V.

Supplements	Iodine no.	Amount per day
Ethyl esters of saturated acids	7.54	85.5 mg
Methyl oleate	88.1	84.2 mg
Ethyl linoleate	170.0	78.7 mg
Ethyl linolenate	231.0	85.5 mg
Ethyl arachidonate	265.5	52.6 mg
Pyridoxine	--	25 gamma
Pantothenic acid (calcium salt)	--	50 gamma
		Amount per week
Vitamin A		250 I U
Vitamin D		100 I U

Table 3. The effect of supplements on rat's fat.

Dietary Supplements	Whole rat	Carcass-fat			Liver-fat	
	Change in	Mixed fatty acids			Mixed fatty acid	
	Mean wt.	Mean wt.	%	I_2-Value	Mean wt.	%
	gm	gm			gm	
I. Without pyridoxine and calcium pantothenate						
Ethyl arachidonate	-1.3	1.18	1.78	92.5	67.0	1.30
Ethyl linolenate	+5.1	2.10	2.66	95.3	51.2	1.37
Ethyl linoleate	+7.1	2.09	2.72	69.0	40.3	1.13
Methyl oleate	+1.4	2.18	2.96	92.7	40.3	1.04
Control	-5.5	1.35	1.94	89.3	39.2	1.12
II. With pyridoxine and calcium pantothenate						
Ethyl arachidonate	+74.0	15.92	9.65	66.0	77.2	1.00
Ethyl linolenate	+73.7	17.55	11.01	75.0	117.3	1.66
Ethyl linoleate	+71.7	16.25	11.15	69.4	62.7	0.77
Methyl oleate	+60.0	16.74	10.16	71.0	133.2	1.84
Ethyl esters of sat. acids	+60.7	14.23	9.06	66.0	75.2	1.01

Table 4. Spectrophotometric studies of mixed fatty acids from carcasses of rats on various supplements.

Dietary supplements	Saturated acid		Oleic acid		Linoleic acid		Arachidonic acid	
	%	Mg*	%	Mg*	%	Mg*	%	Mg*
I. Without pyridoxine and calcium pantothenate								
Ethyl arachidonate	15.61	276	77.10	1375	1.10	19.6	6.20	112
Ethyl linolenate	13.44	350	77.81	2070	4.44	118.0	5.31	142
Ethyl linoleate	14.54	395	80.00	2120	2.11	57.6	3.00	93
Methyl oleate	15.16	435	76.15	2180	3.16	90.8	5.53	158
Control	17.95	348	75.35	1460	0.35	2.6	6.80	113
II. With pyridoxine and calcium pantothenate								
Ethyl arachidonate	33.30	3120	63.32	6110	1.44	130.0	1.92	186
Ethyl linolenate	28.07	3100	68.50	7560	1.35	149.0	2.08	230
Ethyl linoleate	37.92	4220	57.35	6400	3.43	383.0	1.30	134
Methyl oleate	25.54	2000	72.20	7340	1.25	125.0	1.05	106
Ethyl esters of sat. acids	30.76	3040	67.72	6670	0.86	86.9	0.74	73

Note: * indicates mg of the fatty acids in 100 gm of rat's carcasses.

Table 5. Spectrophotometric studies of mixed fatty acids from the livers of rats on various supplements.

Dietary supplements	Saturated and Oleic acids		Linoleic acid		Arachidonic acid	
	%	Mg*	%	Mg*	%	Mg*
I. Without pyridoxine and calcium pantothenate						
Ethyl arachidonate	51.16	660	14.02	182	34.82	453
Ethyl linolenate	60.11	810	16.02	306	23.87	388
Ethyl linoleate	52.71	590	18.41	205	28.88	324
Methyl oleate	76.37	786	10.12	105	13.51	140
Control	72.80	815	10.63	119	16.57	185
II. With pyridoxine and calcium pantothenate						
Ethyl arachidonate	55.07	670	10.90	116	35.53	370
Ethyl linolenate	63.44	686	14.44	240	22.12	334
Ethyl linoleate	56.33	434	14.11	109	29.46	226
Methyl oleate	77.28	1422	8.37	54	11.25	207
Ethyl esters of sat. acids	80.81	821	7.58	77	11.61	118

Note: * indicates mg of the fatty acid in 100 gm of rat's livers. No linolenic acid was present except for traces in the rats' livers on supplements of methyl oleate or ethyl esters of sat. acids with pyridoxine and pantothenic acid.

Table 6. The effect of supplements on rat's fat.

Dietary supplements	Whole rat	Carcass-fat			Liver-fat	
	Change in	Mixed fatty acids			Mixed fat acid	
	mean wt.	Mean wt.	%	I_2 values	Mean wt.	%
Ethyl linolenate + pyridoxine						
+calcium pantothenate	+75.7 gm	17.56 gm	11.01	73.0	117.5 mg	1.66
Ethyl linolenate + pyridoxine	+24.5 gm	7.30 gm	6.58	75.0	74.0 mg	1.82
Ethyl linolenate alone	+0.7 gm	2.10 gm	2.66	95.9	51.8 mg	1.60

Table 7. Spectrophotometric studies of mixed fatty acids from carcasses of rats on various supplements.

Dietary supplements	Saturated acid		Oleic acid		Linoleic acid		Arachidonic acid	
	%	Mgs	%	Mgs	%	Mgs	%	Mgs
Ethyl linolenate + pyridoxine								
+calcium pantothenate	28.07	5100	68.30	7860	1.38	149.0	2.05	230
Ethyl linolenate + pyridoxine	24.75	1622	69.00	4520	2.01	101.0	3.38	280
Ethyl linolenate alone	13.44	398	77.81	3070	4.44	118.0	5.21	142

Table 6. Spectrophotometric studies of mixed fatty acids from the livers of rats on various supplements.

Dietary supplements	Saturated and oleic acids		Linoleic acid		Arachidonic acid	
	%	Mgs	%	Mgs	%	Mgs
Ethyl linolenate + pyridoxine + calcium pantothenate	53.44	885	14.44	240	32.12	534
Ethyl linolenate + pyridoxine	62.87	1010	14.07	288	23.06	384
Ethyl linolenate alone	59.11	810	15.02	206	25.37	355

Table 9. The effect of supplements on mean weight of rat's tissues.

Dietary supplements	Carcass tissue	Liver tissue
	gm	gm
I. Without pyridoxine and calcium pantothenate		
Ethyl arachidonate	66.4	4.6
Ethyl linolenate	79.0	3.0
Ethyl linoleate	76.9	3.8
Methyl oleate	72.4	3.9
Control	68.7	3.3
II. With pyridoxine and calcium pantothenate		
Ethyl arachidonate	164.7	7.3
Ethyl linolenate	150.9	7.1
Ethyl linoleate	163.8	6.9
Methyl oleate	154.8	7.2
Ethyl esters of saturated acids	144.6	7.4
III. With ethyl linolenate		
Pyridoxine + calcium pantothenate	158.9	7.1
Pyridoxine	107.6	4.9
Control	79.0	3.8

EXPLANATION OF PLATE I

Effect of dietary supplements on the growth of rats.

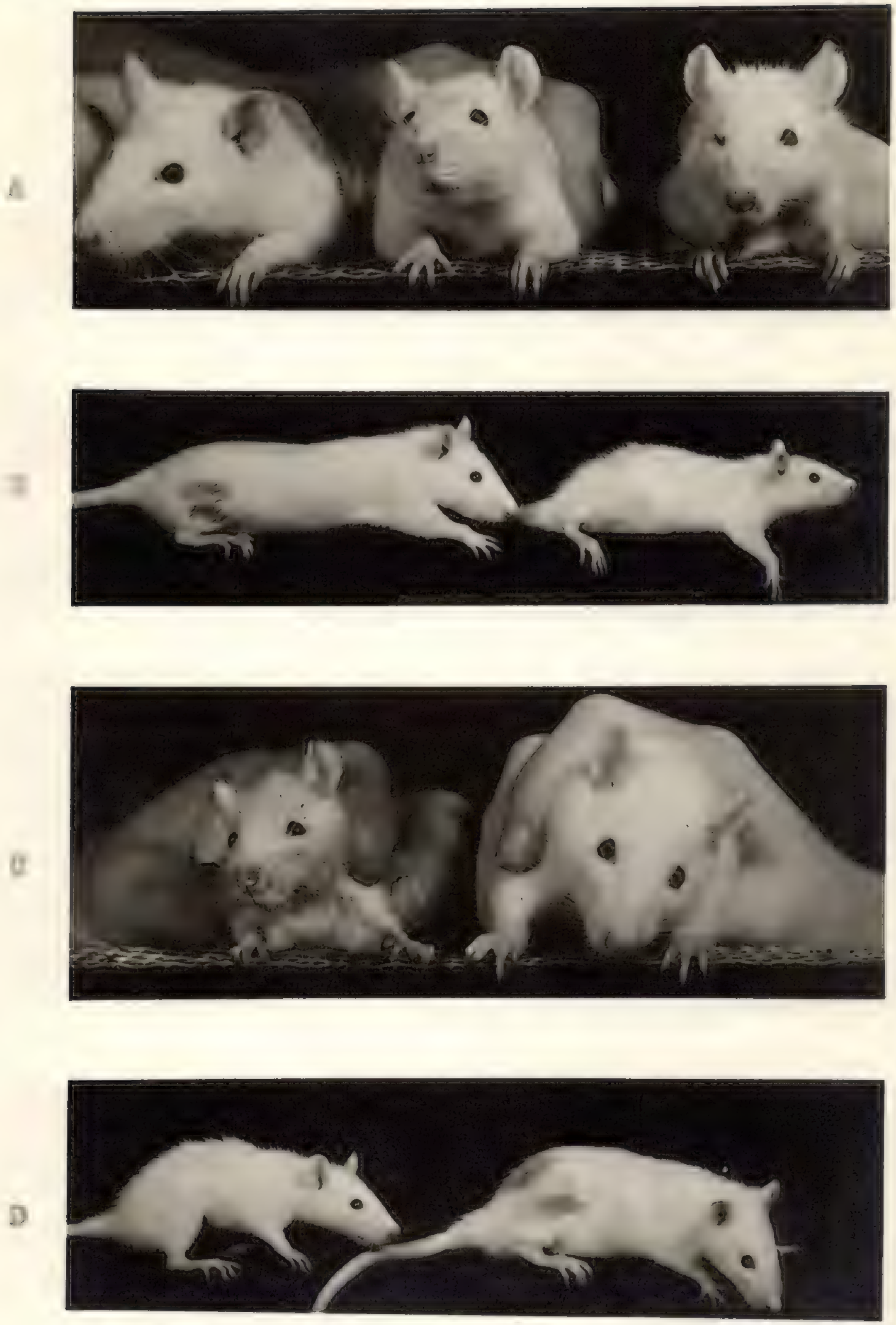
A. Left to right: Ethyl linolenate, pyridoxine and calcium pantothenate; ethyl linolenate and pyridoxine; ethyl linolenate alone.

B. Left to right: Ethyl linoleate, pyridoxine and calcium pantothenate; ethyl linoleate alone.

C. Left to right: Methyl oleate alone; methyl oleate, pyridoxine and calcium pantothenate.

D. Left to right: Control group; ethyl esters of the saturated fatty acids of cottonseed oil, pyridoxine and calcium pantothenate.

PLATE 1



DISCUSSION

Both pyridoxine and unsaturated fatty acids were reported by Birch (3) to be necessary for the cure of the acrodynia-like dermatitis of rats. In the present experiment, although the animals which had been fed with ethyl linolenate without pyridoxine and calcium pantothenate stayed alive, they showed some acrodynia symptoms and did not gain in weight. The addition of pyridoxine promoted growth. The amount of mixed fatty acids in the carcass tissues was increased while their iodine values decreased. Both pyridoxine and unsaturated fatty acids are therefore necessary for the metabolism of fat in the animal.

If the dietary supplement contained pantothenic acid in addition to pyridoxine and ethyl linolenate, the animals exhibited a further increase in body weight, more mixed fatty acids were found to be deposited in the carcass tissue, while their iodine values were further lowered. Novelli and Lipmann (16) observed that pantothenic acid in the form of coenzyme A was concerned with the primary attack on acetate, presumably the condensation with oxalacetate in the Krebs cycle. Thus it might be said that both pyridoxine and pantothenic acid are functioning in the metabolism of unsaturated fatty acids and should be present in the diets of the animals for normal growth.

When the animals which received pyridoxine and pantothenic acid in the diet were compared to those which did not receive these supplements, the former were found to contain more saturated and oleic acids in the carcass tissues, while the amounts of linoleic and arachidonic acids were not appreciably changed. The increase in the amount of saturated fatty acids deposited was, however, much greater than that of oleic acid, and thus the iodine values of the mixed fatty acids were lowered and the percentage of unsaturation seemed to be decreased.

The composition of the mixed fatty acids in the liver tissues remained the same as long as the animals received one of the essential fatty acids in their diets. The composition was not influenced by the presence or absence of pyridoxine and pantothenic acid in the diet.

According to Bloor (4), the fat of the liver represents mostly food or depot fat temporarily stored there on the way to be used in metabolism. Such loosely stored fat might be expected to be readily moved either in or out of the liver under varying conditions. This ease of mobilization of fats might explain the constant composition of fatty acids in the liver of rats. The liver was found to contain about 1.30 percent of mixed fatty acids. The latter contained approximately 60 percent of saturated and oleic acids, 15 percent of linoleic acid and 30 percent of arachidonic acid.

The interconversion of fatty acids in vivo were studied by Stetten and Schoenheimer (30) by feeding the rats with the fatty acids labeled with deuterium. These authors came to the conclusion that the rat can and does continuously convert palmitic acid into several other fatty acids in its body tissues in the following manner:

$$\begin{tikzcd}
\text{Stearic} \arrow[r, shift left] \arrow[d, shift right] & \text{Palmitic} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & \text{Shorter chain acids} \arrow[l, shift left] \\
\text{Oleic} \arrow[u, shift right] & \text{Palmitoleic} \arrow[u, shift right] &
\end{tikzcd}$$

However, they showed that linoleic or other highly unsaturated fatty acids could not be formed from palmitic or oleic acid. In the present experiment, it seems that linoleic and arachidonic acids were interconvertible. More linoleic acid was found in the rats which received arachidonic acid and vice versa. Similar observations have been reported by Nunn and Smedley-Maclean (17), Ellis and Isbell (11), Eckstein (10), Spadolla and Ellis (30) and Rieckehoff, Holman, and Burr (24).

Randolph (25) suggested that linolenic acid on entering the animal body was immediately converted into linoleic and arachidonic acids. This author also indicated that linolenic acid was present only in animals which died during the assay period. In agreement with these results, both the carcass and liver tissues of the animals in the present experiment did not contain linolenic acid. Thus linolenic acid likely would be converted into arachidonic and linoleic acids, but no evidence was found for the conversion of the latter into linolenic acid.

In the liver tissues of rats which did not receive any fatty material or those fed with methyl oleate or the ethyl esters of saturated fatty acids, some arachidonic and linoleic acids were deposited, though much less than the amounts in the animals which received the essential fatty acid. This seemed to be in disagreement with the observations of Nunn and Smedley-Maclean (17), who reported that unless linoleic or linolenic acid is given, the rat is unable to synthesize the C_{20} or C_{22} acid containing four or more double bonds. The presence of small amounts of arachidonic and linoleic acids might be explained by the fact that the animals deposited certain amounts of those fatty acids in their tissues during the first three weeks of their growth on the normal diets, or that the animals possessed these fatty acids at birth. Recently, Rieckehoff, Holman, and Burr (24) indicated that the tissue fatty acids of the rat retain considerable amounts of polyunsaturated fatty acids on a fat free diet.

Comparison of the amount of ingested fats to the amount of fat deposited in the tissues clearly shows that they are far in excess of the amount of fat consumed. This is specially true when pyridoxine and pantothenic acid were present in the diet. These excess amounts of fats must be synthesized from other sources such as protein or carbohydrates. Longenecker (15) demonstrated that carbohydrate or protein produced a body fat in the rat, possessing an iodine value of 60 to 65 with the major component fatty acids being oleic, palmitic, and hexadecenoic acids. This generalization might be applied here, since the fatty acids deposited in the carcass tissues did contain more saturated and oleic acids. It appeared therefore that pyridoxine and pantothenic acid, besides their effect in the metabolism of unsaturated fatty acids, also were functional in the conversion of carbohydrates and proteins into fats.

SUMMARY

Rats on fat deficient diets were supplemented with esters of the essential or nonessential fatty acids with and without pyridoxine and pantothenic acid. The results showed that in the absence of pyridoxine and pantothenic acid, less arachidonic and linoleic acids were found in the liver tissues in animals supplemented with nonessential fatty acids. All the animals survived throughout the experimental period of 5 weeks, but they did not show any appreciable increase in body weight.

When the animals were supplemented with pyridoxine and pantothenic acid in addition to the ester of the fatty acids, they grew normally and increased rapidly in weight. Thus pyridoxine and pantothenic acid were confirmed to be effective in the metabolism of the unsaturated fatty acids.

The fatty acid composition of the liver tissues were analyzed and found to remain constant, and independent of the influence of pyridoxine and pantothenic acid in the diet. In general, it was shown that the liver tissues contained larger percentages of arachidonic and linoleic acids than the carcass tissues.

Both the carcass and liver tissues were found free of linolenic acid. From this it was postulated that the rats did

not deposit any linolenic acid in their tissues. It was further postulated that the linolenic acid fed was converted into arachidonic, linoleic, and other fatty acids.

Pyridoxine and pantothenic acid were shown to play a role in the synthesis of fats from carbohydrates and protein besides functioning in the metabolism of unsaturated fatty acids.

ACKNOWLEDGMENT

The writer wishes to express his sincere appreciation to Dr. Fred A. Kummerow, Major Instructor in the Department of Chemistry, for suggesting this problem for thesis work and for directing this study throughout the course of his research, also to those fellow students and members of the faculty who made helpful suggestions and who assisted in numerous ways during the course of the experiment.

REFERENCES

(1) Anderson, W. E., and L. B. Mendel.
The relation of diet to the quality of fat produced in the animal body. Jour. Biol. Chem. 76: 729-747. 1928.

(2) Barnes, R. H., W. O. Lundberg, H. T. Hanson, and G. O. Burr.
The effect of certain dietary ingredients on the keeping quality of body fat. Jour. Biol. Chem. 149: 313-322. 1943.

(3) Birch, T. W.
The relation between vitamin B_6 and the unsaturated fatty acid factor. Jour. Biol. Chem. 124: 775-793. 1938.

(4) Bloor, W. R.
Biochemistry of the fatty acids. New York: Reinhold Publishing Corporation, 1943.

(5) Brice, B. A., M. L. Swain, B. B. Schaeffen, and W. C. Ault.
Detection of small portions of polyunsaturated constituents in fatty materials. Oil and Soap 22: 219-224. 1945.

(6) Brown, J. B., and G. Y. Shinowara.
Studies on the chemistry of the fatty acids: II. the preparation of pure oleic acid by a simplified method. Jour. Amer. Chem. Soc. 59: 6-8. 1937.

(7) Burr, G. O.
Significance of the essential fatty acids. Federation Proc. 1: 224-233. 1942.

(8) Burr, G. O., and M. M. Burr.
A new deficiency disease produced by the rigid exclusion of fat from the diet. Jour. Biol. Chem. 82: 345-367. 1929.

(9) Burr, G. O., and M. M. Burr.
On the nature and role of the fatty acids essential in nutrition. Jour. Biol. Chem. 86: 587-621. 1930.

(10) Eckstein, H. C.
The influence of diet on the body fat of the white rat. Jour. Biol. Chem. 81: 613-628. 1929.

(11) Ellis, N. R., and H. S. Isbell.
The influence of the character of the ration upon the composition of the body fat of hogs. Jour. Biol. Chem. 69: 219-238. 1926.

(12) Kummerow, F. A., J. Hite, and S. Kloxin.
Fat rancidity in eviscerated poultry. Poultry Science 27: 689-694. 1948.

(13) Hume, E. M., L. C. A. Nunn, I. Smedley-MacLean, and H. H. Smith.
Studies of the essential unsaturated fatty acids in their relation to the fat deficiency disease of rats. Biochem. Jour. 32: 2162-2177. 1938.

(14) Lipmann, F.
Chem. and Engin. News 26: 860. 1948.

(15) Longenecker, H. E.
Deposition and utilization of fatty acids. Jour. Biol. Chem. 128: 645-658. 1939.

(16) Novelli, G. D., and Lipmann, F.
The involvement of coenzyme A in acetate oxidation in yeast. Jour. Biol. Chem. 171: 833-834. 1947.

(17) Nunn, L. C. A., and I. Smedley-MacLean.
The nature of the fatty acids stored by the liver in the fat-deficient disease of rats. Biochem. Jour. 32: 2178-2184. 1938.

(18) Oleson, J. J., and S. Black.
Factor W and pantothenic acid in the nutrition of the rat. Jour. Biol. Chem. 133: lxxiii-lxxiv. 1940.

(19) Quackenbush, F. W., F. A. Kummerow, and H. Steenbock.
Effectiveness of linoleic, arachidonic and linolenic acids in reproduction and lactation. Jour. Nutr. 24: 213-224. 1942.

(20) Quackenbush, F. W., B. B. Platz, and H. Steenbock.
Rat acrodynia and the essential fatty acids. Jour. Nutr. 17: 115-126. 1939.

(21) Quackenbush, F. W., and H. Steenbock.
Body fats in rat acrodynia. Jour. Nutr. 24: 393-398. 1942.

(22) Quackenbush, F. W., H. Steenbock, F. A. Kummerow, and B. R. Platz.
Linoleic acid, pyridoxine, and pantothenic acid in rat dermatitis. Jour. Nutr. 24: 225-234. 1942.

(23) Randolph, P. M.
The metabolism of linolenic acid. Unpublished M. S. thesis, Kansas State College, Manhattan, Kansas, 1949.

(24) Rieckehoff, I. G., R. T. Holman, and G. O. Burr.
Polyethenoid fatty acid metabolism: effect of dietary fat on polyethenoid fatty acids of rat tissues. Arch. of Biochem. 20: 331-340. 1949.

(25) Rollett, A.
Zur kennitus der linolsaure. Z. Physiol. Chem. 62: 410-421. 1909.

(26) Salmon, W. D.
The supplementary relationship of vitamin B_6 and unsaturated fatty acids. Jour. Biol. Chem. 133: lxxxiii. 1940.

(27) Schneider, H., H. Steenbock, and B. R. Platz.
Essential fatty acids, vitamin B_6, and other factors in the cure of rat acrodynia. Jour. Biol. Chem. 132: 539-551. 1940.

(28) Schoenheimer, R., and D. Rittenberg.
Deuterium as an indicator in the study of intermediary metabolism. Jour. Biol. Chem. 113: 505-510. 1936.

(29) Spadola, J. M., and N. R. Ellis.
The effect of the ingestion of cottonseed oil before and after hydrogenation on the composition of the body fat of the rats. Jour. Biol. Chem. 113: 205-218. 1936.

(30) Stetton, DeWitt Jr., and R. Schoenheimer.
The conversion of palmitic acid into stearic and palmitoleic acids in rats. Jour. Biol. Chem. 133: 329-345. 1940.

(31) Turpeinen, O.
Further studies on the unsaturated fatty acids essential in nutrition. Jour. Nutr. 15: 351-366. 1938.

(32) Yasuda, M.
The determination of the iodine number of lipids. Jour. Biol. Chem. 94: 401-409. 1931.

Date Due